Caldicoprobacter oshimai gen. nov., sp. nov., an anaerobic, xylanolytic, extremely thermophilic bacterium isolated from sheep faeces, and proposal of *Caldicoprobacteraceae* fam. nov.

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An obligately anaerobic, xylanolytic, extremely thermophilic bacterium, strain JW/HY-331^T, was isolated from sheep faeces collected from a farm at the University of Georgia, USA. Cells of strain JW/HY-331^T stained Gram-positive and were catalase-negative, non-motile rods. Single terminal endospores (0.4–0.6 µm in diameter) swelled the mother cell. Growth ranges were 44–77 °C (optimum 70 °C at pH^{70 °C} 7.2) and pH^{70 °C} 5.9–8.6 (optimum 7.2 at 70 °C). Salt tolerance was 0–2.0% (w/v) NaCl. No growth was observed at or below 42 °C or at or above 79 °C or at pH^{70 °C} 5.7 and below or 8.9 and above. In the presence of 0.3% yeast extract and 0.1% tryptone, strain JW/HY-331^T utilized xylose, glucose, galactose, cellobiose, raffinose and xylan as carbon and energy sources, but not dextran, soluble potato starch, CM-cellulose, cellulose powder, casein or Casamino acids. Fermentation products from glucose were lactate, acetate, ethanol, CO₂ and H₂. The G+C content of the genomic DNA was 45.4 mol% (HPLC). Major cellular fatty acids were iso-C_{17:0}, iso-C_{15:0} and anteiso-C_{17:0}. No respiratory quinones were detected. The cell-wall structure was a single layer (Gram-type positive) of the peptidoglycan type A1γ; the cell-wall sugars were galactose and mannose. Based on 16S rRNA gene sequence analysis, '*Catabacter hongkongensis*' HKU16 (85.4% similarity), *Caloramator fervidus* ATCC 43204^T (84.2%) and *Caloranaerobacter azorensis* MV1087^T (83.4%) were the closest relatives, but they were only distantly related to strain JW/HY-331^T. On the basis of physiological, chemotaxonomic and phylogenetic data, isolate JW/HY-331^T (=DSM 21659^T =ATCC BAA-1711^T) is proposed as the type strain of *Caldicoprobacter oshimai* gen. nov., sp. nov., placed in *Caldicoprobacteraceae* fam. nov. within the order *Clostridiales* of the phylum *Firmicutes*.

Several moderately thermophilic, anaerobic bacteria with growth optima at 50–65 °C have been isolated from animal faeces. Examples include *Clostridium thermocellum* from cow and horse manure, *Moorella thermoacetica* from horse faeces and *Thermoanaerobacter thermocopriae* from camel faeces (Wagner & Wiegel, 2008). However, comparatively little is known about the occurrence of anaerobic extreme thermophiles, with growth optima above 65 °C, in animal faeces. A study has reported the presence of an anaerobic extreme thermophile, *Caldanaerobacter subterraneus*, in cow faeces (Yokoyama *et al.*, 2007a), implying that dormant extreme thermophiles might prevail in animal faeces. Many anaerobic thermophiles can degrade hemicellulosic material, the second most abundant component of plant fibre; however, the other extremely thermophilic

hemicellulolytic bacteria were not isolated from animal faeces but from hot springs or oil-producing wells (Wagner & Wiegel, 2008).

Samples of sheep, horse, pig and cow faeces were collected at different locations on farms of the University of Georgia, USA, and were stored anaerobically at 4 °C until use. Basal medium I consisted of (per litre distilled water) 0.25 g Na₂PO₄·7H₂O, 0.1 g NH₄Cl, 0.18 g MgCl₂·6H₂O, 10 mg FeCl₂·4H₂O, 1 mg MnCl₂·4H₂O, 0.2 g NaCl, 0.2 g KCl, 1 mg resazurin, 1 g cysteine hydrochloride and 40 mM HEPES. For enrichment cultures, basal medium I was supplemented with 0.2% yeast extract, 0.1% tryptone, 0.5% birchwood xylan and 0.5% beechwood xylan and adjusted to pH^{70 °C} 7.0 (Wiegel, 1998) with 10 M NaOH. Approximately 0.5 g mixed faeces was inoculated into 10 ml aliquots of the anaerobic medium. After 1 week of incubation at 74 °C, 1 ml was transferred into fresh

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JW/HY-331^T is AB450762.

medium and incubated for a further week. Only the sample inoculated with the sheep faeces yielded growth. The transfer procedure was repeated a further three times. Pure cultures were obtained by the modified Phytigel-shake roll-tube technique (Ljungdahl & Wiegel, 1986) using 5 % Phytigel and 0.12 % $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and basal medium I supplemented with 0.25 % yeast extract, 0.1 % tryptone, 0.05 % xylose, 0.5 % birchwood xylan and 0.5 % beechwood xylan. White to creamy, round colonies (0.5–1.3 mm in diameter) developed after 8 days of incubation at 70 °C. Strain JW/HY-331^T was obtained by three rounds of single-colony isolation.

Basal medium II (pH^{70 °C} 7.2), containing (per litre distilled water) 54 mg $\text{Na}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g NH_4Cl , 0.18 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 4 mg $\text{Fe}(\text{NH}_4)\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 1.5 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 g NaCl, 0.2 g KCl, 1 mg resazurin, 1 g cysteine hydrochloride and 20 mM HEPES, was used in morphological analyses. For correct pH measurement under the applied conditions and the superscript notation see Wiegel (1998). For light microscopy, cells of strain JW/HY-331^T were cultured in liquid xylan medium (0.5 % yeast extract, 0.25 % tryptone, 0.25 % birchwood xylan and 0.25 % beechwood xylan). The cells were mounted on agarose-coated glass slides and observed with a Vonox phase-contrast microscope (Olympus). For transmission electron microscopy (TEM), cells cultured in a glucose medium (0.5 % yeast extract, 0.25 % tryptone and 0.5 % glucose) were ultrathin-sectioned and stained with 2 % uranyl acetate and a lead staining solution (Sigma-Aldrich). The sections were observed with a JEM-1200 EX (JEOL). Vegetative cells were straight to curved rods, 0.4–0.5 µm in diameter and 4–14 µm long, and appeared singly or in pairs (Fig. 1a). No active mobility was detected. Sporulating cells were observed in the stationary growth phase. Single spherical endospores (0.4–0.6 µm in diameter) were formed in a swollen terminus (Fig. 1b). In the death phase, the swollen termini separated from the rods (Fig. 1c) and the spores were subsequently released from the swollen body. Many cells failed to form a spore and became round, spheroplast-like cells. Based on microscope observations of cells transferred into fresh media, it appeared that the spheroplast-like cells were not viable. Vegetative cells stained Gram-positive, and the cell-wall structure was of a single layer (Gram-type positive), as judged by TEM (Fig. 1d, e). Analyses of cell-wall peptidoglycan by TLC and GC (MacKenzie, 1987; Schleifer & Kandler, 1972) revealed the presence of the amino acids alanine, glutamic acid and *meso*-diaminopimelic acid (molar ratio 1.0:1.0:1.1), indicating the peptidoglycan type A1γ. The cell-wall sugars were galactose and mannose, as determined by TLC (Staneck & Roberts, 1974).

Until otherwise stated, the glucose medium (mentioned above) was used for the physiological analyses. Strain JW/HY-331^T was obligately anaerobic, with no growth in pink (=oxidized resazurin) medium, and was negative for both catalase and oxidase. The temperature range for growth at

Fig. 1. Morphology of strain JW/HY-331^T. (a–c) Phase-contrast micrographs of vegetative cells (a), sporulating cells (b) and a released spore (c). (d) TEM image of ultrathin-sectioned cells. (e) Enlargement of (d), showing the cell-wall structure. Bars, 10 µm (a), 5 µm (b), 2 µm (c), 500 nm (d) and 50 nm (e).

pH^{70 °C} 7.2 was 44–77 °C, with an optimum of 70 °C (no growth at or below 42 °C or at or above 79 °C), and the pH^{70 °C} range for growth at 70 °C was 5.9–8.6, with optimum growth at pH^{70 °C} 7.2 (no growth at pH^{70 °C} 5.7 or below or at pH^{70 °C} 8.9 or above). Salinity tolerance was 0–2.0 % (w/v) NaCl (no growth at or above 2.5 %). The shortest doubling time observed was 48 min under optimal growth conditions (70 °C at pH^{70 °C} 7.2). Substrate utilization was analysed using basal medium II supplemented with 0.3 % yeast extract, 0.1 % tryptone and 0.5 % test substrate. No substantial growth was observed in medium containing 0.3 % (w/v) yeast extract and 0.1 % (w/v) tryptone without the addition of another substrate; however, yeast extract was required for measurable growth with xylose, glucose, galactose, fructose, mannose, cellobiose, lactose, raffinose and xylan from birchwood, beechwood and oat spelt. Growth on all three xylans was

confirmed by measuring the resulting fermentation products lactate, acetate and ethanol and the intermittent accumulation of the hydrolysis product xylose. No growth was observed with dextran, soluble potato starch, CM-cellulose, cellulose powder, cellulose (filter paper), sorbitol, mannitol, lactate, acetate, ethanol, butanol, peptone, casein, gelatin or Casamino acids. In the presence of 0.3 % yeast extract and 0.1 % tryptone and using a gas phase of 70 % H₂ and 30 % CO₂, no autotrophic growth was observed. No growth enhancement (or formation of the corresponding reduced products) was observed with the possible electron acceptors 20 mM Na₂SO₄ (sulfide), 5 or 2.5 mM Na₂SO₃ (sulfide), 20 mM Na₂S₂O₃ (sulfide or sulfur), 20 mM NaNO₃ (nitrite) or 5 mM NaNO₂ (ammonium, N₂). Fermentation end products from glucose were quantified using basal medium II supplemented with 0.3 % yeast extract, 0.1 % tryptone and 0.5 % glucose. Approximately 0.6 mol lactate, 0.3 mol acetate, 0.5 mol ethanol, 0.8 mol CO₂ and 0.4 mol H₂ were detected per mol glucose degraded. Without the addition of glucose to the medium, only traces of the fermentation products were observed.

The DNA G + C content was 45.4 mol%, as determined by HPLC (Mesbah *et al.*, 1989). For the analysis of cellular fatty acids, fatty acid methyl esters were prepared from a freeze-dried sample and analysed by GC (Guckert *et al.*, 1985). The major cellular fatty acids (>10 %) were branched, saturated fatty acids with odd numbers of carbon atoms: iso-C_{17:0} (31.1 %), iso-C_{15:0} (23.7 %) and anteiso-C_{17:0} (12.9 %). Minor cellular fatty acids (>1 %) were iso-C_{17:0} 3-OH (8.1 %), C_{16:0} (6.8 %), anteiso-C_{15:0} (5.7 %), iso-C_{16:0} (2.4 %) and C_{17:0} 2-OH (1.9 %). Trace cellular fatty acids (<1 %) were iso-C_{11:0}, anteiso-C_{11:0}, iso-C_{13:0}, C_{14:0}, iso-C_{15:0} 3-OH, C_{16:0} 3-OH, iso-C_{16:0} 3-OH, C_{17:0} 2-OH, C_{18:0}, iso-C_{18:0}, C_{18:1}ω7c, C_{18:1}ω9c, iso-C_{19:0}, iso-C_{16:0} 3-OH and C_{20:4}ω6,9,12,15c. No respir-

atory quinones were detected by a combination method of TLC and HPLC (Tindall, 1990a, b).

A nearly complete 16S rRNA gene sequence was amplified by PCR with primers 27F and 1492R (Lane, 1991) and sequenced by Macrogen (Seoul, South Korea). Sequences of the closest relatives were retrieved by a BLAST search and aligned by using the program CLUSTAL W (Thompson *et al.*, 1994). The distance matrix was calculated by the Jukes-Cantor model (Jukes & Cantor, 1969) using MEGA 4 (Tamura *et al.*, 2007). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). Phylogenetic analysis using the 16S rRNA gene sequence revealed that strain JW/HY-331^T belongs to the order *Clostridiales*; however, strain JW/HY-331^T does not group within any family within the order *Clostridiales* with a validly published name. Interestingly, strain JW/HY-331^T showed the highest identity, 99.5 %, to an unidentified clone OTU4, which was detected in enrichment cultures at 75 °C from cow faeces collected in Japan (Yokoyama *et al.*, 2007b) (Fig. 2). Based on their similar source of isolation and 16S rRNA gene sequence, the unidentified clone OTU4 and strain JW/HY-331^T belong to the same species, which presumably prevails in herbivore faeces. Strain JW/HY-331^T also showed high similarity to unidentified clones Hb (97.7 %) and LNE-5 (95.7 %) (Hobel *et al.*, 2004). These unidentified clones were respectively detected in thermophilic enrichment cultures inoculated with bioreactor sludge and a sample from a hot spring. Among isolates with effectively and/or validly published names, strain JW/HY-331^T showed the highest identities to '*Catabacter hongkongensis*' HKU16 (85.4 %) (Lau *et al.*, 2007), *Caloramator fervidus* ATCC 43204^T (84.2 %) (Patel *et al.*, 1987) and *Caloranaerobacter azorensis* MV1087^T (83.4 %) (Wery *et al.*, 2001). Both *Caloramator fervidus* and *Caloranaerobacter azorensis* belong to the family *Clostridiaceae*, whereas '*Catabacter hongkongensis*' is cur-

Fig. 2. Neighbour-joining tree generated using 16S rRNA gene sequences, showing the relationships of strain JW/HY-331^T to its closest relatives and the type species of type genera for families within the order *Clostridiales*. Numbers at nodes represent bootstrap percentages (above 50 %) from 1000 replicates. Bar, 0.05 substitutions per nucleotide position.

rently proposed to belong to a novel family 'Catabacteriaceae', consisting of a single genus and single species, within the order Clostridiales. 'Catabacter hongkongensis', isolated from a clinical blood sample, is described as a non-spore-forming mesophile, significantly different from strain JW/HY-331^T in its physiological characteristics. Both *Caloramator fervidus* and *Caloranaerobacter azorensis* are anaerobic thermophiles, similar to strain JW/HY-331^T (Table 1). However *Caloramator fervidus*, isolated from a hot spring, differs from strain JW/HY-331^T in Gram staining, DNA G+C content and morphology. *Caloranaerobacter azorensis*, isolated from a deep-sea vent, differs from strain JW/HY-331^T in salinity requirement for growth, spore formation, cell-wall structure and DNA G+C content.

In the 16S rRNA gene sequence-based phylogenetic tree, strain JW/HY-331^T forms a separate branch within the order Clostridiales, and clusters only with unidentified clones (Fig. 2). Strain JW/HY-331^T is only distantly related to the single member of the family 'Catabacteriaceae', but the identity to the proposed type strain of the species 'Catabacter hongkongensis' is only 85.4 %, suggesting that it belongs to a different family. Strain JW/HY-331^T shows even lower similarities (76–81 %) to members of all other type genera of the families within the order Clostridiales. On the basis of these physiological, chemotaxonomic and phylogenetic data, strain JW/HY-331^T is proposed as the type strain of the novel taxon *Caldicoprobacter oshimai* gen. nov., sp. nov.; *Caldicoprobacter* gen. nov. is proposed as the type genus for the novel family *Caldicoprobacteraceae* fam. nov. within the order Clostridiales of the phylum Firmicutes.

Description of *Caldicoprobacter* gen. nov.

Caldicoprobacter (Cal'di.co'pro.bac'ter. L. adj. *calidus* hot; Gr. n. *kopros* dung, N.L. masc. n. *bacter* rod; N.L. masc. n. *Caldicoprobacter* a rod from dung growing at elevated temperatures).

Cells are Gram-staining positive, spore-forming, non-motile, straight to curved rods. Negative for catalase and oxidase. Thermophilic. Neutrophilic, with a pH range for growth of approx. pH 5–9. Cellular fatty acids are composed mainly of 15- and 17-carbon-containing saturated, branched fatty acids (iso-C_{17:0}, iso-C_{15:0} and anteiso-C_{17:0}). The peptidoglycan type is A1γ. Obligately anaerobic chemo-organotrophs. The type species is *Caldicoprobacter oshimai*.

Description of *Caldicoprobacter oshimai* sp. nov.

Caldicoprobacter oshimai (o.shi'ma.i. N.L. gen. masc. n. *oshimai* of Oshima, named after the Japanese microbiologist Tairo Oshima, in honour of his many contributions to our knowledge of thermophiles and their biochemistry).

Displays the following properties in addition to those described for the genus. Cells are 0.4–0.5 µm in diameter and 4–14 µm long and appear singly or in pairs. Main cell-wall sugars are galactose and mannose. Spherical endospores (0.4–0.6 µm in diameter) are formed in a terminal position, causing swelling of the mother cell. Non-sporulating cells form protuberances in the stationary growth phase that then become round, spheroplast-like cells that are not viable. Extremely thermophilic, growing at 44–77 °C (optimum 70 °C) at pH^{70 °C} 7.2 (no growth at or below 42 °C or at or above 79 °C), and neutrophilic, with a pH^{70 °C} range for growth at 70 °C of 5.9–8.6 and an optimum at pH^{70 °C} 7.2 (no growth at or below pH^{70 °C} 5.7 or at or above pH^{70 °C} 8.9). Tolerates 0–2.0 % (w/v) NaCl. Xylose, glucose, galactose, fructose, mannose, cellobiose, lactose, raffinose and xylan are utilized as carbon and energy sources but not dextran, soluble potato starch, CM-cellulose, cellulose powder, cellulose (filter paper), sorbitol, mannitol, lactate, acetate, ethanol, butanol, peptone, casein, gelatin or Casamino acids. Fermentation end products from glucose are lactate, acetate, ethanol, CO₂ and H₂. The DNA G+C content of the type strain is 45.4 mol%.

Table 1. Phenotypic comparison of strain JW/HY-331^T with its closest relatives with validly published names

Strains: 1, JW/HY-331^T; 2, *Caloramator fervidus* ATCC 43204^T (data from Patel *et al.*, 1987); 3, *Caloranaerobacter azorensis* MV1087^T (Wery *et al.*, 2001). +, Positive; –, negative; ND, no data available. All strains are positive for xylan utilization.

Characteristic	1	2	3
Length of rods (µm)	4–12	2–5	0.5–2
Motility	–	Weak	+
Gram stain	+	–	–
Cell-wall structure	Single layer	Single layer	Double layer
Spore formation	+	+	–
DNA G+C content (mol%)	45.4	39	27
Temperature range (optimum) (°C)	44–77 (70)	43–74 (68)	45–65 (65)
pH ^{70 °C} range (optimum)	5.9–8.6 (7.2)	5.5–8.5 (7–7.5)	5.5–9.0 (7)
NaCl range (% w/v)	0–2.0	ND	0.65–6.5
Starch utilization	–	+	+

The type strain, JW/HY-331^T (=DSM 21659^T =ATCC BAA-1711^T), was isolated from sheep faeces collected from a farm at the University of Georgia, USA.

Description of *Caldicoprobacteraceae* fam. nov.

Caldicoprobacteraceae (Cal'di.co'pro.bac'te.ra'ce.ae. N.L. masc. n. *Caldicoprobacter* type genus of the family; suff. -aceae ending to denote a family; N.L. fem. pl. n. *Caldicoprobacteraceae* the *Caldicoprobacter* family).

Includes spore-formers exhibiting Gram-type-positive, rod-shaped cells with anaerobic chemo-organotrophic metabolism. The DNA G+C content is around 45 mol%. Belongs to the order *Clostridiales* within the class '*Clostridia*' of the phylum *Firmicutes*. The type genus is *Caldicoprobacter*.

Acknowledgements

We thank Weidong Zhao and Katherine E. A. Segarra for the biogas analysis, Peter Schuman for the cell-wall analysis, Brian Tindall for the quinone analysis, Takamasa Hanaichi and Kaoru Akiyama at the Hanaichi Ultrastructure Research Institute, Japan, for the TEM analysis and Jean Euzéby for help with the Latin nomenclature. The Identification Service of the DSMZ (Braunschweig, Germany) performed chemotaxonomic analyses including the G+C mol% determination.

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